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THE OPTIMAL TTC DOSE AND ITS CHEMICAL REDUCTION LEVEL DURING SOIL DEHYDROGENASE ACTIVITY ASSAY

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A b s t r a c t. 2,3,5-triphenyltetrazolium chloride (TTC) is a dye largely used for determination of microbiological activity in soil samples by dehydrogenase assay. TTC is colourless in the oxidised form and red when reduced, due to formation of the product 1,3,5-triphenyl formazan (TPF). In this study, different doses of TTC ranging from 0.2 to 3.0% were added to six different soil types with the aim to verify the effect of chemical TTC reduction and to optimise the substrate dose in order to prevent a toxic effect on microorganisms. Regarding the technical simplicity and cost-effectiveness, the TTC assay is commonly recommended as an efficient method for determination of soil biological activity, provided that an appropriate concentration is applied, depending on the soil type. The lowest TTC levels of 0.2 and 0.5% are recommended for Albic Luvisol and Eutric Fluvisol, respectively, and the highest (2%) for Brunic Arenosol and Haplic Luvisol soils. It has also been demonstrated that DHA has biological origin in more than 90%, whilst chemical TTC reduction accounts for less than 10%, and therefore does not interfere with the correctness of the results obtained.

K e y w o r d s: dehydrogenase activity, soil, TTC dose, chemical TTC reduction

INTRODUCTION

Soil enzyme activities can be regarded as effective indicators of soil quality changes resulting from environmental stress or management practices (Utobo and Tewari 2014). Soil dehydrogenases (EC 1.1.1.1) are the major representatives of the oxidoreductase enzyme class tightly linked with microbial oxidoreduction processes. Importantly, dehydrogenases do not accumulate extracellularly in the soil; they occur intracellularly in all living microbial cells and thus are considered to be an indicator of overall soil microbial activity (Das and Varma 2011, Wolińska and Stępniewska 2012, Januszek *et al*. 2014). In other words, when dehydro-

genase is present in the soil, the presence of bacteria can reasonably be concluded (Walls-Thumma 2000). The concept of determining the metabolic activity of microorganisms in soil and other habitats by measuring dehydrogenase activity (DHA) was introduced by Lenhard (1956). However, being an indicator is connected with meeting a number of the following conditions (Utobo and Tewari 2014): it has to be easy to measure, sensitive to system changes, integrative, and able to predict changes. In that context, determination of DHA in soil samples under laboratory conditions should be precise and not burdened with the risk of errors. One of possible disruptions occurring during soil DHA measurements is the phenomenon of chemical (not biological) substrate reduction.

The most common laboratory procedure used for DHA determination is the method developed by Casida *et al.* (1964). According to this method, specific dyes such as 2,3,5-triphenyltetrazolium chloride (TTC) that can specify the flow of electrons are useful indicators of the electron transport system (ETS) activity (Wolińska and Stępniewska 2012, Januszek *et al*. 2014). By the reduction of TTC, i.e. a stable, colourless, water soluble heterocyclic organic salt, an insoluble, high-colour red triphenyl formazan (TPF) product is formed and can be quantified calorimetrically (Wolińska and Stępniewska 2012). The added artificial substrate (TTC) has two functions: (a) it makes organic materials more available to microorganisms and (b), at the same time, the bacteria convert it to TPF which can be extracted from the soil and analysed (Alef and Nannipieri 1995, Walls-Thumma 2000). Only live microorganisms could reduce TTC by enzymatic action (dehydrogenase assay) and the resulting TPF is kept inside granules in cells which become red (Beloti *et al.* 1999). Generally, one unit of DHA is the amount of the enzyme that would reduce 1 µmol of TTC to 1 µmol of TPF per minute (Mahmoud and Ghaly 2004). Hence, DHA is regarded to be a suitable biological test confirming the presence of living microorganisms in the investigated material (Januszek *et al*. 2014). However, Friedel *et al.* (1994) described several disadvantages of TTC reduction, i.e. inhibition by the presence of oxygen, toxicity to microorganisms, and low reaction activity which requires a long incubation period (30 h). The study performed by Praveen-Kumar and Tarafdar (2003) indicated that while 70-100% of soil bacteria and actinomycetes are capable of using the TTC as an electron acceptor, this percentage falls to around 5% in respect to soil fungi. Ever wider interest is evoked by the new generation of water soluble tetrazolium salts (WST-1). Ishiyama *et al*. (1993) reported that the positively charged tetrazolium salts are intracellulary reduced with the use of NADH, whereas negatively charged WST-1 are probably reduced on the cell's surface, however the source of NADH is completely different. Other authors (Trevors 1984, von Mersi and Schinner 1991) suggested using another tetrazolium salt instead of TTC, and recommended iodonitrotetrazolium chloride (INT) as less toxic to microorganisms and more rapidly reduced. However, INT exhibits lower solubil-

ity in water than TTC and, consequently, yields values that are considerably lower in comparison with the TTC method (Friedel *et al.* 1994). Thus, to alleviate the effect of soil reaction to DHA, optimal conditions have been provided by using buffers with pH values between 7.0 and 8.0 (von Mersi and Schinner 1991). Additionally, besides pH optimisation, we also emphasised that a proper TTC dose – not toxic to microorganisms – has to be used, and the substrate dose concentration should be selected according to the soil type. Consequently, we hypothesised that the universal (3%) TTC dose is not always appropriate and depends on the soil type; thus, it is necessary to optimise the TTC dose according to the investigated soil material.

The toxic effect of TTC on microorganisms was indicated in a limited number of studies. Friedel *et al.* (1994) reported that the optimal TTC concentration, evaluated separately for arable, forest, and grassland soils, ranged from 0.4 to 3.0%. However, Gong (1997) tested substantially higher TTC doses ranging from 3.2 to 12.5% and noted that in the case of loamy soil under flooded conditions (30°C) DHA reached the maximal value as a result of application of 12.5% TTC, whereas at 37°C the dose of 3.2% TTC was optimal. Mahmoud and Ghaly (2004) studied the effect of pH and temperature on non-enzymatic TTC reduction and found that TTC could be reduced non-enzymatically at temperatures higher than 75°C and/or under alkaline pH conditions (9.5-11.0).

In literature database, there is no precise information about the phenomenon of chemical TTC reduction, which may strongly affect the DHA test credibility and its biological character. Thus, the aim of the study was to determine both the optimal (nontoxic to microorganisms) TTC dose and its chemical reduction level in respect to six different soil types (FAO): Eutric Histosol, Haplic Luvisol, Mollic Gleysol, Eutric Fluvisol, Albic Luvisol, Brunic Arenosol.

MATERIALS AND METHODS

Soils description

Six different soils, representative of the main Polish soil types, were selected for the study. Four samples, representing the SE part of Poland and Lubelskie voivodeship (51°13′N 22°54′E), classified according FAO as Eutric Histosol, Haplic Luvisol, Mollic Gleysol, Eutric Fluvisol, were collected from the topsoil (0-20 cm) during spring 2014. Extractions of the soils from the agriculturally managed surface layer were performed using Egner's bow. Three replicates of 0.5 kg, consisting of ca. 50 random samples, were taken from a 100 m^2 area and combined into one sample in order to receive the most representative soil material. The two other soil units (Albic Luvisol, Brunic Arenosol), also taken from

the surface horizon and representing the NW part of Poland and Zachodniopomorskie voivodeship (53°36′N 15°32′E), were derived from the resources of the Bank of Soil Samples (BSS) belonging to the Institute of Agrophysics PAS in Lublin (Bieganowski *et al*. 2013). The soils were diverse in terms of total carbon (TC), pH, and redox potential (Eh). pH ranged from 5.63 to 6.72, Eh from 395.8 to 647.8 mV, and TC from 0.02 to 39.46% (Table 1).

Table 1. Chemical soil properties

Soil type	Location of sampling site	pH (H_2O)	Eh (mV)	TC (%)
Eutric Histosol	Orłowskie peatland, Poleski National Park (SE Poland)	6.17	460.0	39.46
Haplic Luvisol	Stary Gaj, Lubelskie voivodeship (SE Poland)	6.47	647.8	0.02
Mollic Gleysol	Kosiorów, Lubelskie voivodeship (SE Poland)	5.63	466.5	28.95
Albic Luvisol	Niemcze, Koszalinskie voivideship (NW Poland)	6.72	403.8	0.98
Brunic Arenosol	Sztumska Wieś, Elbląskie voivodeship (NE Poland)	6.10	395.8	13.50
Eutric Fluvisol	Kępa Solecka, Lubelskie voivodeship (SE Poland)	6.73	442.8	1.40

The pH and Eh were determined from a 2:1 soil suspension in distilled water, using a multifunctional potential meter pIONneer 65 (Radiometer Analytical S.A., France). The measurements were taken in triplicate after stabilisation of the readings.

Total carbon (TC) was determined using an automatic carbon analyser TOC-V_{CSH} SSM 5000A (Shimadzu, Japan). Soil samples (150 mg) were pulverised, dried prior to analysis, and then combusted at 900°C in a column containing a platinum and cobalt oxide catalyst. Under the conditions mentioned, all carbon compounds were converted into carbon dioxide and detected by an infrared detector. Each TC recording was carried out in triplicate.

Dehydrogenase activity assay

In order to determine DHA, the method proposed by Casida *et al*. (1964) was applied. Accordingly, dyes such as triphenyltetrazolium chloride (TTC), which can determine the flow of electrons, are useful indicators of the activity of the electron transport system. By the reduction of TTC, which is a stable, colourless, water soluble heterocyclic organic salt, an insoluble, high-colour red triphenyl formazan (TPF) product is formed and can be quantified calorimetrically at the

range of visible light (485 nm). Briefly, if the red colours of soil samples prepared for spectrophotometric analyses are more intensive, the measured level of DHA is higher (Wolińska and Stępniewska 2012). Consequently, soil samples without red colours or those with light red colours are characterised by lower DHA values.

The soil samples (6 g) mixed with distilled water (4 ml) and calcium carbonate – $CaCO_3$ (120 mg) were left to react with the TTC solution at 30°C for 20 h and then they were extracted with ethanol and incubated for 1h in the dark. Absorbance (λ = 485 nm) was measured using a UV-1800 (Shimadzu) instrument. DHA was expressed as μg TPF g^{-1} min^{-1}. All measurements were performed in triplicate and calculated on the basis of the oven-dry (105°C) soil mass (Wolińska and Bennicelli 2009).

Determination of TTC optimal dose

The optimal TTC dose in regard to the six soil types was determined based on the concentration range suggested by Małachowska-Jutsz and Miksch (2010), and in accordance with the ISO 23753 policy provisions (2005). In the current study, the following concentrations of TTC were tested: 0.2; 0.5; 1.0; 1.5; 2.0, and 3.0%. It is worth emphasising that a TTC concentration of 3% is recommended for use in the most popular method developed by Casida *et al.* (1964) for DHA measurement in soil samples.

Soil samples were prepared analogously to the standard analytical protocol (Casida *et al*. 1964), i.e., 4 ml of distilled water, 120 mg of calcium carbonate, and 1 ml of TTC at an appropriate concentration (0.2-3%) were added to 6 g of soil. Then, the samples were incubated (20 h, 30°C), extracted with ethanol (1 h, darkness), and filtered. Absorbance (λ = 485 nm) was measured using a UV-1800 (Shimadzu) instrument. Six series of analyses were performed, separately for each soil type and each TTC concentration. Each analysis was carried out in triplicate.

Determination of TTC chemical reduction

Soil samples (6 g) with $CaCO_3$ (120 mg) were placed in 50 ml Erlenmayer flasks and subjected to sterilisation (Hiclave Hg-50, HMC). Two sterilisation treatments were applied: (a) single soil autoclaving (1 h, 121°C) and (b) twofold soil autoclaving (1 h, 121°C) – with maintenance of a one-day interval, allowing a possible resurgence of microbial life resistant to high temperature conditions. Next, 3% TTC (1 ml) and distilled water (4 ml) were added. The samples were incubated (20 h, 30°C), extracted with ethanol (1 h, darkness), and filtered. Absorbance (λ = 485 nm) was measured using a UV-1800 (Shimadzu) instrument. TPF concentrations were read from the calibration curve (Fig. 1).

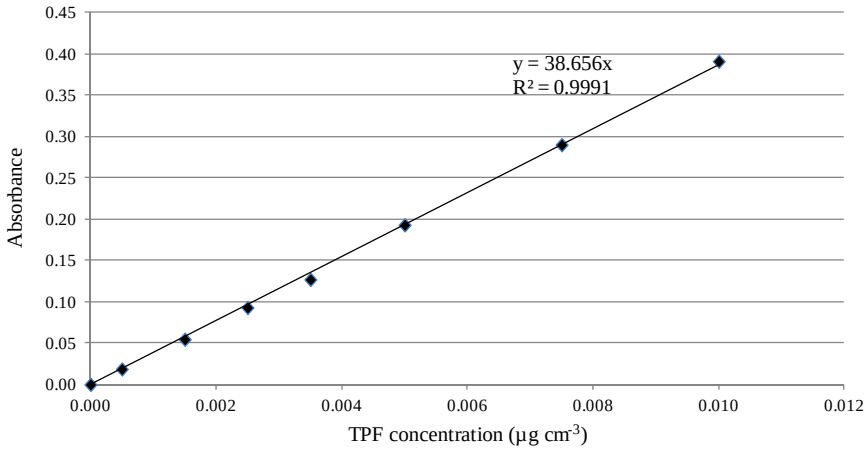

Fig. 1. Calibration curve for TPF calculation

RESULTS

Optimal TTC dose

The optimal doses of TTC concentration determined for the six soil types are shown in Figure 2. We demonstrated that the optimal TTC content differs depending on the soil type.

The maximum values of absorbance indicated that the optimum TTC dose amounted to 0.2% for Albic Luvisol and 0.5% for Eutric Histosol and Eutric Fluvisol. However, in the case of Eutric Histosol, no significant difference ($p > 0.05$) was found between the TTC 0.5 and 1.0% doses, even though absorbance decreased by 9.4% after the application of 1.0% TTC in comparison with the 0.5% dose.

Analogically, no significant differences ($p > 0.05$) were observed in Eutric Fluvisol at the TTC concentration between 0.2 and 0.5%, despite the fact that the absorbance level noted at the 0.2% TTC dose was lowered by 23% in relation to the 0.5% dose. The 1% TTC concentration proved to be optimal for Mollic Gleysol and was confrmed by the maximum absorbance level. Nevertheless, the differences in absorbance observed between the TTC concentrations of 0.5-1.5% were not significant ($p > 0.05$), even though absorbance was reduced by 17.8% in regard to its maximum. The TTC concentration between 1.5 to 2.0% was estimated as optimal for the Brunic Arenosol soil type, whereas the dose of 2.0% TTC should be applied in the Haplic Luvisol soil (Fig. 2).

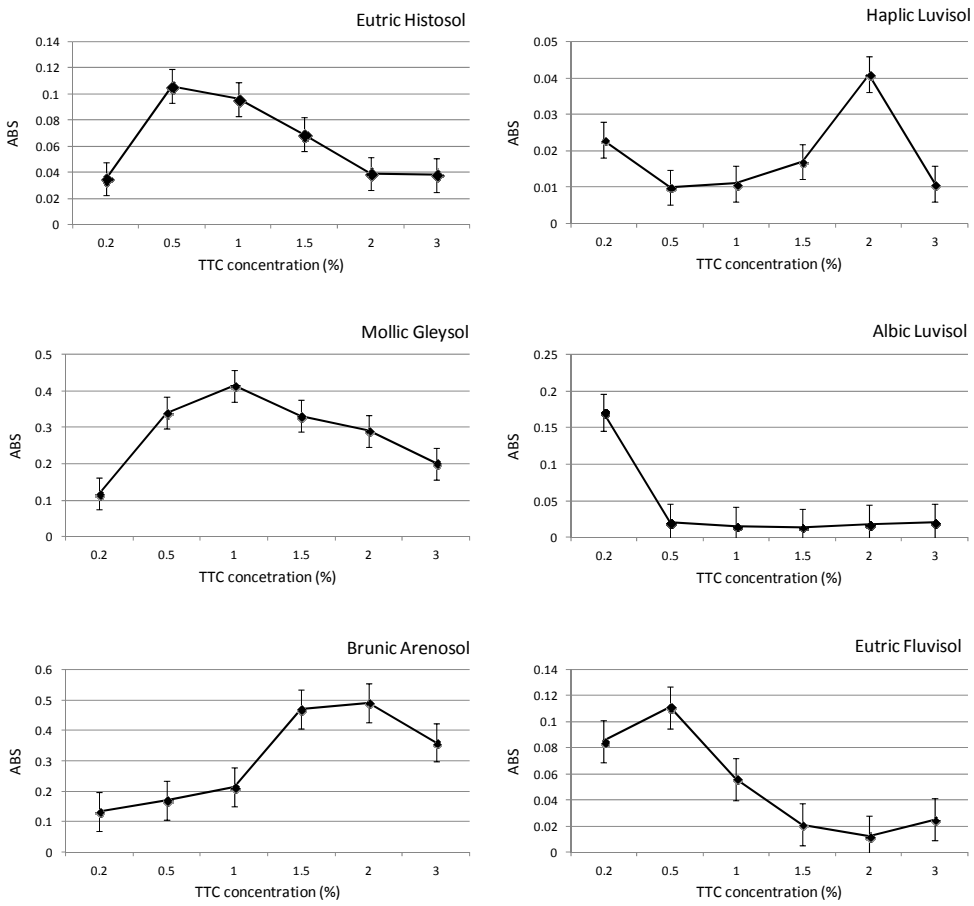

Fig. 2. Optimal TTC concentration for each of the soil types, determined at λ = 485 nm. Mean values of three replicates with standard error (SE) are presented

Biological and chemical TTC reduction

In order to assess the level of chemical TTC reduction that may affect the correctness and reliability of the results obtained during standard DHA measurements, soils subjected to the single and twofold autoclaving process were examined. The amount of TPF, i.e. the result of dehydrogenase action in natural (not autoclaved) soil extracts, and the TPF concentration obtained in the soil samples after the single and twofold sterilisation process is presented (Table 2).

Table 2. TPF produced on biological and chemical way with percent of chemical *vs.* biological TTC reduction in soils after single and twofold autoclaving cycles

Soil type	TPF concentration (µg cm^{-3})			Percent of chemical TTC reduction in respect to biological reduction	
	Biologically produced	Chemically produced after single autoclaving cycle	Chemically produced after twofold auto-claving cycle	Single soil autoclaving (%)	Twofold soil autoclaving (%)
Eutric Histosol	0.00264865	0.0000620	0.00000780	2.34	0.29
Haplic Luvisol	0.00000028	0.00000011	0.0000000018	3.90	0.61
Mollic Gleysol	0.00525144	0.00034492	0.000010011	6.55	1.90
Albic Luvisol	0.00340611	0.00025969	0.000003400	7.59	0.10
Brunic Arenosols	0.00936465	0.00028456	0.00001302	3.03	0.14
Eutric Fluvisol	0.00533290	0.00023382	0.000006901	4.37	0.13

As shown by the presented data, the biological reduction of TTC to TPF is a decidedly dominating process in the soil environment under laboratory conditions during DHA estimation.

The amount of TPF is a measure of dehydrogenase activity. In other words, a higher level of TPF confirms higher DHA in the soil. Similarly, the lower the TPF formation, the lower DHA is noted. The highest TPF concentration, and thus DHA, was noted in Brunic Arenosol. In contrast, the lowest product content was found in *Haplic Luvisol*. Mollic Gleysol and Eutric Fluvisol were characterised by a similiar TPF level (0.0052-0.0053 µg cm^{-3}), whereas in the case of Eutric Histosol and Albic Luvisol the TPF concentration amounted to 0.0026 and 0.0034 µg cm^{-3}, respectively. As indicated by the data presented above, in soils subjected to a single autoclaving cycle, it is possible to obtain a 2.3-7.6% TPF concentration in comparison to natural (non-sterilised) soils.

Nevertheless, to minimise this undesirable effect, soil samples should be subjected to twofold autoclaving cycles with maintenance of a one-day interval between the sterilisation processes to allow a possible resurgence of bacterial cells that have not been completely destroyed during the single autoclaving process. After the twofold soil autoclaving process with an interval of 24 h, negligible amounts of TPF derived from its chemical reduction, varying in the range of 0.1-1.9%, were noted (Table 2).

DISCUSSION

Optimal TTC dose

Determination of an optimal TTC dose before analysis was considered necessary (Małachowska-Jutsz and Miksch 2010), which confirmed our hyphothesis that the universal (3%) TTC dose is not always appropriate and depends on the soil type (more precisely on the microbial community inhabiting soil); thus, it is necessary to optimise the TTC dose according to the investigated soil material. An optimal substrate dose should be balanced, as it depends on the current composition and physiological state of microorganisms inhabiting studied soils (Małachowska-Jutsz and Miksch 2010). In the presence of bacteria, TTC is reduced to red TPF, which is directly proportional to viable active cells (Moussa *et al*. 2013). Thus, the estabilished TTC concentration should ensure such conditions that the substrate will be able to penetrate intracellular structures (which have dehydrogenase activity) in proper quanitities and will not exert a toxic effect on living microorganisms (Małachowska-Jutsz and Miksch 2010). Various authors have reported poor DHA results when TTC is used as a substrate (von Mersi and Schinner 1991; Friedel *et al*. 1994; Gong 1997; Kumar *et al*. 2013). In our opinion, this fact might be connected with using the universal (3%) TTC concentration instead of an optimised substrate dose with respect to the soil type. When the TTC concentration applied to the soil sample is too high and toxic to microorganisms, no reliable results from the DHA test will be obtained.

In the current study, among the soils investigated, Albic Luvisol, Eutric Fluvisol, and Eutric Histosol, where the optimum substrate dose amounted to 0.2-0.5%, seemed to be the most sensitive to TTC. This value is significantly (ca. 83-93%) lower than the 3% dose suggested by Casida *et al*. (1964). In regard to the Luvisol and Fluvisol soil type, Friedel *et al*. (1994) determined the optimal TTC concentration at the level of 0.8-1%, respectively. For Mollic Gleysol, 1.0% TTC was determined as an optimal dose, whereas higher substrate doses ranging from 1.5 to 2.0% were recommended for Brunic Arenosol and Haplic Luvisol, respectively. Małachowska-Jutsz and Miksch (2010) suggested a TTC concentration from 0.5 to 0.7% for loamy soil (pH = 6.5).

Chemical TTC reduction during standard dehydrogenase assay

It has been demonstrated that the amount of chemically formed TTC strictly depended on the soil type. In the current experiment, Mollic Gleysol proved to be the most “resistant” to the sterilisation procedure, as even after the twofold autoclaving cycles the reaction product, i.e. TPF formed in the chemical process, amounted to 1.9% in comparison with its level produced via the biological process

in the non-autoclaved soil. In all of the other soil types, the chemical TTC reduction after the application of the twofold autoclaving procedures was very low and did not exceed 1%, remaining at a level of 0.1% for Albic Luvisol, Brunic Arenosol and Eutric Histosol, 0.29% for Eutric Histosol, and 0.61% in the Haplic Luvisol. Therefore, the results obtained in the experiment clearly confirmed the fact that the DHA determined under laboratory conditions is of biological origin; it is composed of living microorganisms and is a reliable test for demonstrating their presence in the soil material tested. This could be especially useful in the so-called rapid biological assays applied for biotechnology purposes and aimed at the primary diagnosis of the soil material evaluated for microbiological testing.

Even the slight TPF concentration established in the sterilised soil samples is a proof of chemical TTC reduction, which is in accordance with the observations conducted by Januszek *et al*. (2007). Other authors (Mahmoud and Ghaly 2004) noted that changes in the pH of the sample affected the chemical reduction of TTC. Although no chemical reduction was observed in acidic media (pH < 7), high pH values caused chemical reduction of TTC (Mahmoud and Ghaly 2004; Ghaly and Mahmoud 2006). Our study clearly showed that chemical TTC reduction is possible even at pH < 7 (Table 2), as the pH of the investigated soils ranged from 5.63 to 6.73. In contrast, Januszek *et al*. (2007) concluded that the pH of soil designated for DHA should not be regulated. Moreover, Ghaly and Mahmoud (2006) observed that the TPF yield increased with an increase in the TTC concentration (from 5 to 15 g l^{-1}), pH (from 7 to 9), temperature (from 25 to 55°C) and incubation time (from 1.5 to 4.5 h). They also noted that the TPF yield was more sensitive to changes in temperature, followed by the pH, TTC concentration, and incubation time (Ghaly and Mahmoud 2006).

CONCLUSIONS

1. Our study indicated that one TTC concentration should not be used regardless of the soil type. For the soils investigated in the current study, the following TTC doses are recommended: 0.2% for Albic Luvisol, 0.5% for Eutric Fluvisol and Eutric Histosol, 1% for Mollic Gleysol, and 2% for Brunic Arenosol and the Haplic Luvisol.

2. Chemical reduction of TTC is an accompanying process that always takes place when DHA is determined under laboratory conditions, even at pH < 7. However, its level is usually lower than 10% and amounts to approx. 2.3-7.6%.

3. In order to determine chemical TTC reduction, soil samples should be autoclaved two times, with maintenance of a one-day interval between the sterilisation processes.

4. Control samples should be prepared from twofold autoclaved soils without substrate addition before the sterilisation process. The substrate should be added after the second autoclaving cycle.

5. These findings also confirm the fact that more than 90% of DHA assessed under laboratory conditions is of biological origin. Thus, determination of DHA in soil samples provides a large amount of reliable information about the biological characteristic of soil, provided that an optimal TTC dose is applied.

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OPTYMALNA DAWKA TTC I POZIOM JEJ CHEMICZNEJ REDUKCJI PODCZAS OZNACZEŃ AKTYWNOŚCI DEHYDROGENAZ W GLEBACH

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S t r e s z c z e n i e. 2,3,5-chlorek trifenylotetrazoliowy (TTC) to powszechnie używany barwnik w oznaczeniach mikrobiologicznej aktywności prób glebowych za pomocą testu na aktywność dehydrogenazową. W utlenionej formie TTC jest bezbarwny zaś w zredukowanej przyjmuje barwę czerwoną z powodu wytworzenia się produktu, jakim jest 1,3,5-trifenyloformazan (TPF). W bieżącej pracy różne dawki TTC w stężeniach od 0,2 do 3% dodawano do sześciu różnych typów gleb celem zweryfikowania efektu jego chemicznej redukcji oraz zoptymalizowania dawki substratu tak by nie działała toksycznie na mikroorganizmy. Z uwagi na prostotę i stosunkowo niski koszt oznaczenia, test z użyciem TTC jest powszechnie rekomendowany do oznaczeń aktywności biologicznej gleb, pod warunkiem że będzie stosowana jego właściwa dawka, zależna od typu gleby. Wykazano, że najniższe stężenie TTC 0,2 oraz 0,5% jest właściwe odpowiednio w odniesieniu do gleby płowej spiaszczonej oraz mady rzecznej, podczas gdy wyższa jego dawka (2%) powinna być stosowana w przypadku gleb płowych właściwych (zerodowanych) i gleb płowych typowych. Wykazano ponadto, że aktywność dehydrogenaz w ponad 90% jest biologicznego pochodzenia, zaś chemiczna redukcja TTC stanowi mniej niż 10%, stąd nie wpływa na prawidłowość uzyskiwanych wyników.

S ł o w a k l u c z o w e: aktywność dehydrogenaz, gleba, dawka TTC, chemiczna redukcja TTC